

Neuroprotective maraviroc monotherapy in simian immunodeficiency virus-infected macaques: reduced replicating and latent SIV in the brain

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Objective: HIV-associated neurocognitive deficits remain a challenge despite suppressive combined antiretroviral therapy. Given the association between HIV-induced central nervous system (CNS) disease and replication of HIV in immune-activated macrophages, CCR5 antagonists may attenuate CNS disease by modulating inflammatory signaling and by limiting viral replication.

Design: To establish whether initiating CCR5 inhibition during early infection altered CNS disease progression, outcomes were compared between simian immunodeficiency virus (SIV)-infected macaques treated with maraviroc (MVC) versus untreated SIV-infected macaques.

Methods: Six SIV-infected rhesus macaques were treated with MVC monotherapy for 5 months beginning 24 days postinoculation; 22 SIV-infected animals served as untreated controls. SIV RNA levels in plasma, cerebrospinal fluid, and brain, and CNS expression of TNF α and CCL2 were measured by qRT-PCR. Immunostaining for CD68 and amyloid precursor protein in the brain was measured by image analysis. Plasma sCD163 was measured by ELISA.

Results: SIV RNA and proviral DNA levels in brain were markedly lower with MVC treatment, demonstrating CCR5 inhibition reduces CNS replication of SIV and may reduce the CNS latent viral reservoir. MVC treatment also lowered monocyte and macrophage activation, represented by CNS CD68 immunostaining and plasma sCD163 levels, and reduced both TNF α and CCL2 RNA expression in brain. Treatment also reduced axonal amyloid precursor protein immunostaining to levels present in uninfected animals, consistent with neuroprotection.

Conclusion: CCR5 inhibitors may prevent neurologic disorders in HIV-infected individuals by reducing inflammation and by limiting viral replication in the brain. Furthermore, CCR5 inhibitors may reduce the latent viral reservoir in the CNS. Adding CCR5 inhibitors to combined antiretroviral regimens may offer multiple neuroprotective benefits.

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Introduction

HIV-associated neurocognitive disorders (HAND), including HIV-associated dementia (HAD), minor cognitive/motor disorder (MC/MD), and asymptomatic neurocognitive impairment (ANI) [1], remain a persistent problem for individuals infected with HIV [2–4]. Although combination antiretroviral therapy (cART) has greatly reduced the incidence of dementia, the prevalence of HAND has increased with treated individuals living longer [5]. Despite the frequency of these disorders, the relative contributions of HIV replication, persistent neuroimmune responses, and neurotoxic properties of cART to HAND are not well defined [5–7].

With cART, virus load in plasma and cerebrospinal fluid (CSF) is suppressed in most individuals to less than 50 copies/ml. Even with adherence to therapy, spontaneous viral reactivation can occur with spikes more than 50 copies/ml. Intermittent virus replication in tissues, especially immune-privileged sites like brain, may contribute to deleterious ongoing immune activation. Despite efficacy of cART in suppressing virus replication, latent virus reservoirs persist; proviral DNA in CD4⁺ lymphocyte subsets and macrophages provide long-lived reservoirs.

CCR5 inhibitors are promising anti-HIV drug candidates that block HIV entry into cells and also may offer numerous beneficial central nervous system (CNS) effects. Maraviroc (MVC), which is approved for use in HIV patients, has relatively high CNS penetrance and minimal neurotoxicity versus other antiretroviral drug classes [8,9]. As CCR5-tropic HIV predominates in the CNS and intermittent HIV replication may occur in the brain despite cART, MVC could block infection of new target cells within the CNS. MVC may also dampen immune activation of resident CNS effector cells, including microglia and astrocytes, and decrease recruitment of leukocytes to the CNS. In-vitro studies demonstrated that MVC inhibits chemotaxis of monocytes to many inflammatory mediators including CCL2, implying that MVC may downregulate in-vivo trafficking of monocytes [10]. As elevated CSF:plasma ratios of CCL2 have been associated with HIV/simian immunodeficiency virus (SIV) CNS disease, MVC-mediated reduction in CNS CCL-2 production may reduce recruitment of monocytes across the blood–brain barrier (BBB) [11].

In clinical studies, it will be difficult to determine whether MVC has a role in the eradication of HIV from CNS latent reservoirs; an animal model will be essential to address this issue. The SIV/macaque model is ideal to

measure viral cellular reservoirs including brain [12,13]. In this report, SIV-infected macaques were treated with MVC to evaluate the impact of CCR5 inhibition on SIV-induced CNS disease independent of other antiretroviral therapies.

Materials and methods

Animal studies

Rhesus macaques (*Macaca mulatta*) were inoculated intravenously with the neurovirulent clone SIV/17E-Fr and the immunosuppressive strain SIV/DeltaB670 [14–16]. Treated animals ($n=6$) received maraviroc (MVC; Pfizer, 200 mg PO BID) beginning 24 days post-SIV inoculation and continuing until euthanized 180 days postinoculation to match the mean survival time-point for the comparison group of untreated SIV-infected macaques ($n=22$) [17]. Eight additional macaques served as uninfected, untreated, controls. Animals were perfused with saline at euthanasia to remove blood from the vasculature. Tissue samples were either immersion fixed (STE, Streck, Omaha, Nebraska, USA) or flash-frozen. Animal studies were approved by the Johns Hopkins Institutional Animal Care and Use Committee per Animal Welfare Act regulations and the USPHS Policy on Humane Care and Use of Laboratory Animals.

Quantification of maraviroc by combined liquid chromatography-tandem mass spectrometry

MVC concentrations in CSF and blood plasma were measured via combined liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. A hexadeuterated (²H₆) analog of MVC was employed as the internal standard. Samples were extracted by protein precipitation with methanol and injected directly for LC-MS/MS analysis. Chromatographic resolution of MVC and its internal standard was achieved via gradient elution employing a reversed-phase C18 column. Multiple reaction monitoring (MRM) was used for detection of the analyte and internal standard as follows: MVC, m/z 513.7 > 389.1; ²H₆-MVC, m/z 519.7 > 389.1. The calibration range for the standard curve was linear over the clinically relevant ranges of 2–2,500 ng/ml with a mean multiple correlation coefficient (r^2) of 0.9979.

SIV RNA and SIV DNA measurements

Viral RNA was isolated from 140 μ l of plasma and CSF using the QIAamp Viral RNA Mini kit (Qiagen). Quantification of virion-associated RNA was performed by qRT-PCR as described [18]. To measure SIV DNA levels in brain, genomic DNA was isolated from 50 mg of

tissue (basal ganglia) using the DNeasy Kit Quiagen. One μm of DNA was analyzed in triplicate by qPCR using specific primers and probes for SIV gag and IFN β . Copy numbers of SIV DNA were normalized to copy numbers of IFN β as a single-copy cellular gene.

Measuring viral and cellular gene expression in the brain

Total RNA was isolated from 50 mg of brain tissue (basal ganglia) by RNeasy kit (Qiagen), and treated with RQ1 DNase (Promega). One microgram of purified RNA was analyzed by qRT-PCR using primers and probes for TNF α and CCL2 [19]. PCR reactions were performed in a Chromo4 thermocycler (BioRad) using a Multiplex PCR Mix (Qiagen). Cellular mRNA levels were normalized by 18S ribosomal RNA levels. Quantitation of gene expression was performed using the $\Delta\Delta$ Ct method [20].

Measurements of protein in central nervous system and plasma

Immunostaining for the macrophage marker CD68 in basal ganglia (KP1; DAKO, Carpinteria, California, USA) and amyloid precursor protein (APP) (Clone LN27, Zymed, South San Francisco, California, USA) in corpus callosum was measured by digital image analysis [21]. Plasma sCD163 was measured by ELISA according to protocol (Trillium Diagnostics).

Statistical analysis

SIV-infected macaques treated with MVC ($n = 6$) were compared to a group of SIV-inoculated macaques ($n = 22$) and uninfected control macaques ($n = 8$) [17]. In-vivo and in-vitro statistical inferences were calculated using non-parametric methods. To determine whether MVC treatment altered SIV-induced CNS macrophage immune activation, SIV RNA and DNA levels, and/or plasma sCD163, untreated SIV and MVC-treated SIV groups were compared using the Mann-Whitney test. Statistical significance was defined as a P value of less than 0.05.

Results

Maraviroc levels in plasma and cerebrospinal fluid

MVC levels in plasma and CSF samples were measured by LC-MS/MS at 1, 2, and 4 h posttreatment (Fig. 1). MVC levels in both compartments were similar to those reported in treated humans, exceeding the EC₉₀ for CCR5-tropic wildtype HIV-1 [22]. Of the sampled time points, the highest concentrations of MVC in both plasma and CSF were present at 2 h posttreatment.

Maraviroc reduces plasma and cerebrospinal fluid viral load

MVC blocks entry of HIV and SIV into susceptible cells *in vitro*, but its efficacy in lowering CNS viral replication has not been evaluated. Compared to untreated SIV-

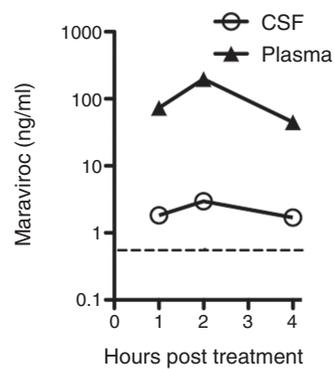


Fig. 1. CSF and plasma levels of maraviroc (MVC) in macaques (group medians, $n = 6$ macaques; 200 mg oral dose) are similar to CSF and plasma levels of MVC reported in treated humans, exceeding the EC₉₀ for R5-tropic wildtype HIV-1. (—represents EC₉₀).

infected macaques, MVC monotherapy reduced median SIV RNA levels in both plasma and CSF at all sample time-points beginning two weeks after treatment initiation (Fig. 2a/b). In MVC-treated macaques, the group median plasma viral load set point was lowered by less than one log compared to untreated animals; however, plasma viral load remained above the limit of detection in all treated SIV-infected macaques over the treatment course. In contrast, the relative decline in SIV RNA in the CSF of MVC-treated animals was greater at most time-points with viral load below the detection limit of 100 copies/mL in three animals at various time-points (Fig. 2b). In one animal, SIV RNA was not detected at any time-point after day 56 p.i. until the terminal time-point. Two additional treated animals did not have detectable SIV RNA in CSF at several time-points.

Maraviroc reduces SIV replication and the latent DNA reservoir in the central nervous system

As CSF does not accurately reflect CNS viral replication, we evaluated SIV replication in brain tissue to determine the direct impact of MVC treatment on CNS. In the basal ganglia, MVC treatment significantly reduced SIV RNA levels versus untreated SIV-infected macaques ($P < 0.001$; Fig. 3a). In five of six treated, SIV-infected animals, SIV RNA levels in the basal ganglia were below the assay limit of detection consistent with marked suppression of CNS viral replication; similarly, vRNA was not detected in the parietal cortex of any MVC-treated animals. Given this marked reduction in SIV replication, to determine whether MVC treatment also reduced the latent SIV reservoir in the CNS, we measured SIV DNA levels in basal ganglia and found that SIV DNA levels also were significantly lower with MVC treatment ($P = 0.0046$; Fig. 3b).

Maraviroc reduces central nervous system inflammation

Ongoing CNS inflammatory responses in the brain may augment SIV replication and contribute to

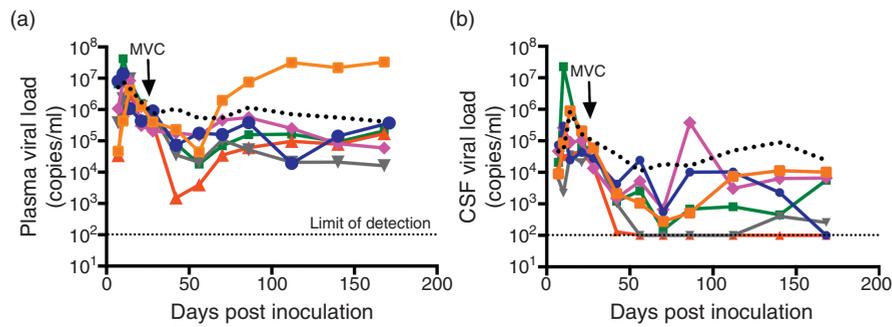


Fig. 2. Treating six SIV-infected macaques with maraviroc alone lowered both plasma (a) and CSF viral loads (b) versus untreated SIV-infected animals (untreated median viral loads represented by dashed black lines) but did not suppress plasma viral replication in any individuals. In contrast, three treated SIV-infected animals had undetectable levels of SIV RNA in CSF at various time points (horizontal line represents limit of detection of assay = 100 copies/ml).

neurodegeneration. Given a role for CCR5 in regulating inflammatory responses via binding of cognate chemokine ligands CCL3–CCL5, we examined whether MVC treatment reduced CNS inflammation in SIV-infected macaques. To determine whether MVC treatment altered macrophage activation in the brain, sections were characterized by immunohistochemistry and digital image analysis. Expression of CD68, a marker of macrophage activation, was reduced by MVC treatment to levels found in uninfected control animals with immunostaining significantly less than untreated SIV-infected animals ($P=0.017$; Fig. 4a).

In the brain, MVC treatment also significantly reduced expression of key pro-inflammatory mediators in the basal ganglia including the cytokine TNF α and the chemokine CCL2 (Fig. 5a and b). Due to the fact that CCR5 inhibition and receptor internalization may have the unintended consequence of upregulating CCR5 expression, we also measured CCR5 levels by qRT-PCR. Although SIV increased CNS CCR5 expression, SIV-infected macaques treated with MVC had CCR5 RNA levels similar to uninfected control animals (Fig. 5c).

Given that sCD163 levels in plasma are associated with neurocognitive impairment in ART treated individuals, we measured plasma sCD163 levels and found that MVC treatment also significantly lowered sCD163 in plasma (Fig. 5d) [23].

Maraviroc prevents neuronal dysfunction

To establish whether MVC therapy prevented neuronal dysfunction, we measured APP levels in the corpus callosum of treated SIV-infected macaques. APP levels in MVC-treated animals were significantly lower than untreated SIV-infected animals ($P=0.001$; Fig. 4b), similar to uninfected control animals.

Discussion

In this study, MVC monotherapy was administered to evaluate the impact of CCR5 inhibition on SIV CNS disease progression independent of the influence of other antiretroviral drugs. In SIV-infected rhesus macaques, MVC monotherapy markedly reduced viral replication in

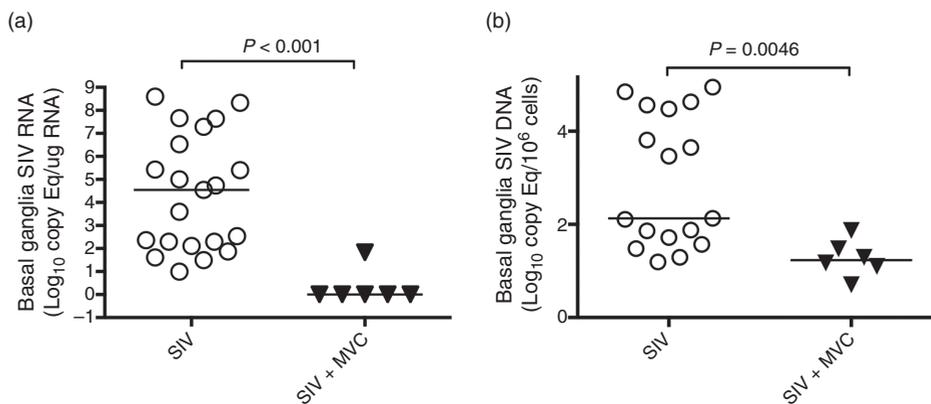


Fig. 3. Maraviroc (MVC) significantly reduced SIV RNA (a) and SIV DNA levels (b) in SIV-infected treated macaques (solid triangles) in the basal ganglia compared to untreated SIV-infected macaques (circles). The impact of MVC on SIV DNA in the CNS (b) illustrates its potential for reducing latent reservoirs in the CNS that will be essential for eradicating HIV from the CNS. Bars represent median values; Mann–Whitney test.

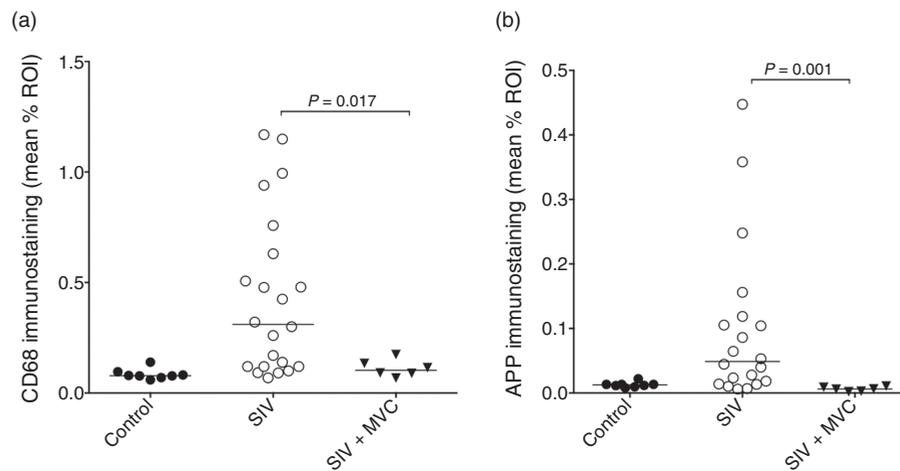


Fig. 4. Treating SIV-infected macaques with maraviroc (MVC) significantly reduced CD68 immunostaining (a) in the basal ganglia compared with untreated SIV-infected macaques (open circles) to levels equivalent to uninfected macaques (closed circles). SIV-infected macaques treated with MVC had significantly reduced APP immunostaining in the corpus callosum compared with untreated SIV-infected macaques (circles) to levels unchanged from untreated SIV-infected macaques. Bars represent median values; Mann–Whitney test.

the CNS, with SIV RNA levels below the limit of detection in the basal ganglia of five of six SIV-infected animals. In addition, brain SIV DNA levels were significantly reduced by MVC treatment, demonstrating that CNS-penetrant MVC may help reduce the CNS latent HIV reservoir in addition to reducing virus replication. MVC is a unique antiretroviral because in addition to blocking HIV entry, it has substantial immunomodulatory effects on chemokine signaling with downstream effects on leukocyte activation and trafficking. Reduced viral replication, decreased CNS leukocyte trafficking, dampened immune activation, and reduced cell–cell virus transmission may contribute to this reduction in CNS SIV DNA observed with CCR5 inhibition [24]. Future studies testing for replication-competent SIV in the CNS after adding MVC to suppressive ART are critical to determine whether combining CNS-penetrant MVC with ART holds promise for virus eradication specifically in the brain.

Despite lower MVC concentrations in CSF versus the periphery, MVC had a greater impact on the relative decline of SIV RNA in the CNS compared with plasma. Similarly, CSF viral RNA levels also were lowered by MVC monotherapy even though CSF viral load does not entirely reflect CNS viral replication. Comparable findings have been reported in HIV-infected individuals receiving adjunctive MVC in which HIV replication in the brain was evaluated indirectly by analyzing CSF samples [25]. Our previous studies using a SIV/pigtailed macaque model of suppressive ART that did not include MVC demonstrated dramatic reductions in SIV RNA in the brain of treated macaques. In those animals, however, SIV DNA levels in brain remained unchanged from untreated SIV-infected macaques. This finding is in sharp

contrast with our findings in macaques treated with MVC monotherapy, further illustrating the potential value of CCR5 inhibition for lowering the latent HIV reservoir in the brain.

Interestingly, in our previous studies using a SIV/pigtailed macaque model of cART, we found evidence of ongoing inflammation in the brain that included persistent elevations of both TNF α and CCL2. These inflammatory markers remained elevated in at least 50% of animals [13]. In contrast, MVC treatment lowered these key pro-inflammatory mediators induced in the CNS by SIV; CNS expression of both TNF α and CCL2 was significantly lower in MVC-treated macaques. The addition of MVC to suppressive cART regimens may downregulate sustained CNS inflammatory responses, reducing recruitment of monocytes into the brain in response to CNS production of CCL2. MVC treatment also significantly reduced CNS immunostaining for CD68, a cellular marker of immune activation in the brain. In addition to inhibiting HIV/SIV-CCR5 interactions, CCR5 inhibition would block binding of the chemokine ligands CCL3–5 to CCR5, thereby also dampening inflammatory signaling. Preventing immune activation of CNS macrophages is likely vital to controlling HAND as activated macrophages are more susceptible to productive infection.

A recent report evaluating the association between ongoing peripheral immune activation of monocyte/macrophages and neurocognitive impairment in HIV-infected individuals receiving suppressive ART found persistently elevated levels of plasma sCD163 in patients with neurocognitive impairment [23]. When coupled with our observation that MVC modulated terminal

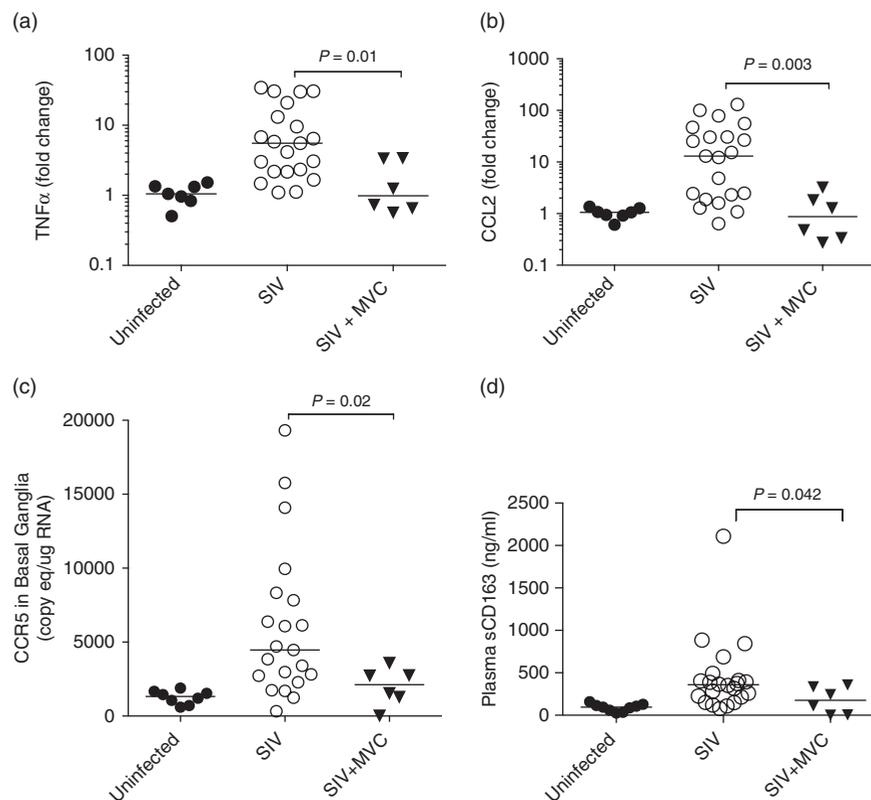


Fig. 5. Treating SIV-infected macaques with maraviroc (MVC) (triangles) significantly reduced expression of TNF α (a) and CCL2 (b) in the basal ganglia compared with untreated SIV-infected macaques (open circles) to levels present in control animal levels (closed circles). Similarly, CCR5 RNA levels in the brain (c) and plasma sCD163 levels (d) were lowered by MVC treatment. Bars represent median values, $\Delta\Delta$ Ct method with Mann–Whitney test (a–c); Mann–Whitney test (d).

plasma sCD163 in SIV-infected macaques, this suggests that CCR5 inhibition may be a valuable adjunct to cART in individuals with neurologic deficits due to its downregulation of peripheral cellular immune responses that have an adverse impact on the CNS. Although measuring different outcomes, other clinical trials suggest that the full immunologic effects of MVC remain to be determined and additional trials are warranted [26].

This study evaluated the impact of MVC monotherapy initiated at an early asymptomatic time-point. At this time-point, a CNS viral reservoir has already been established; therefore, the goal of treatment was not preventing the initial establishment of CNS infection [19]. We have shown that the CNS SIV reservoir is continually archived during asymptomatic infection even in animals receiving early cART (day 12 p.i.) [18,27]. These observations, as well as the impact of CCR5 inhibition on SIV DNA in the brain, suggests that MVC treatment during asymptomatic infection may influence both the character (i.e. viral genotype) and the size of the CNS latent reservoir.

We have previously reported that alterations in neuronal function in SIV-infected macaques are represented by

accumulation of amyloid precursor protein (APP) in axons, a morphologic finding that closely corresponds with impairment of fine motor tasking in SIV-infected macaques [28,29]. Here, we found that SIV-infected macaques treated with MVC had significantly reduced CNS APP immunostaining in the corpus callosum, showing that MVC treatment also reduced SIV-induced neuronal dysfunction. Although MVC treatment may prevent CNS damage, evidence of early damage to the CNS during acute infection in SIV (i.e. before MVC was started in this study) and HIV suggests that MVC may play a beneficial role by limiting or reversing early neuronal damage [19,30]. Tracking alterations in neuronal function in HIV patients receiving MVC intensification by neurocognitive assessments and neuroimaging techniques such as magnetic resonance spectroscopy will confirm whether CCR5 inhibition is neuroprotective in the clinical setting of HAND.

HIV studies of MVC intensification have not reported an improvement in neurologic assessments, however, relatively few infected individuals treated for short times were evaluated [31]. Given our findings, larger-scale studies expanded to include inflammatory and viral

outcome measures will be critical. Barriers to widespread use of CCR5 antagonists, including the requirement to characterize co-receptor usage, potential drug resistance, and emergence of HIV strains utilizing chemokine receptor CXCR4, may be offset by substantial beneficial immunomodulatory dampening of chemokine signaling that sustains immune cell activation and recruitment. The ultimate test of successful eradication will be determination of HIV reactivation in periphery or brain after cessation of cART. Performing such studies in SIV cART models designed to incorporate MVC will be crucial to address this key issue.

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S.E.B.: Performed experiments, data analysis.

K.A.M.P.: Performed experiments, data analysis.

S.E.Q.: Performed experiments, data analysis.

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L.A.: Performed experiments, data analysis.

W.H.: Performed experiments, data analysis.

P.T.T.: Directed statistical analysis, wrote manuscript.

J.L.M.: Conceived study, analyzed data, wrote manuscript.

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Conflicts of interest

There are no conflicts of interest.

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